THE EMBO JOURNAL

Manuscript EMBO-2009-73496

The human core exosome interacts with differentially localized processive RNases: hDIS3 and hDIS3L

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Review timeline:

Submission date:	22 December 2009
Editorial Decision:	12 February 2010
Revision received:	11 May 2010
Editorial Decision:	17 May 2010
Accepted:	18 May 2010

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 February 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. I apologise for the length of time that it has taken to have your manuscript reviewed but this is in part to delays over the holiday period. It has now been seen by three referees whose comments are shown below.

I hope you understand if I do not go through each individual issue raised by the referees, but in general they find the characterization of hDIS3L and hDIS3 and their association with the exosome to be interesting and are supportive of publication in the EMBO Journal pending satisfactory revision. The referees would like to see more definitive evidence for hDIS3L having 3'-5' exonuclease activity and its cytoplasmic localization. Referee #3 also requests an examination of the effects of depletion of the RNAses on cell viability. There are also several controls that should be added.

Should you be able to address these issues, we would be happy to consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This study uses SILAC co-immunoprecipitation analysis to identify hDIS3 and the related hDIS3L protein as part of the human exosome. Furthermore, using multiple antibody and tagged protein reagents and cells, the authors go on to demonstrate distinct differences in the subcellular localization of these factors (along with the RRP6/PM-SCL100 exonuclease). Finally, using siRNA knockdowns and purified proteins, the authors demonstrate a role for all of these factors in exosome function as well as putative endonuclease activity only in the hDIS3 protein.
Overall this is a very interesting study as it identifies likely distinct forms of the mammalian exosome as well as describes for the first time the enzymatic activities of hDIS3 homologs. Given the importance of the exosome in RNA processing and stability, I think that these results will be of significant interest to a broad audience. I do have a few comments/criticisms below to help polish the work as well as better characterize the enzymatic activities.

1. Supplementary Fig. 4: Given that the anti-DIS3L antibody detects a non-specific, ~co-migrating band in western blots (Fig. 3D) as well as innate difficulties in normalizing western blot efficiencies of individual antibodies, it is not clear to me how truly quantitative and thus meaningful is the analysis presented in Fig. S4
2. Fig. 5 and elsewhere - please clearly define the ss17-A14 and related substrates for the reader
3. Figs. 5. S5 and S6: If I understand the experiment correctly, these studies were performed with 5' labeled RNA substrates. Therefore this assay alone does not rule out endo activities (or 5'-3' exonuclease activity for that matter). While I think these alternative activities are unlikely given what has been previously published in yeast, nonetheless this is the first time the activities of these two human proteins will be presented. Thus I feel it is important for the authors to clearly demonstrate for the reader that they are revealing a 3'-5' exonuclease. For example, does a 3' structure block the nuclease of the protein? Does the protein have activity on circular substrates?
4. Fig. 5B: The data using the hDIS3L-TAP is not very convincing. In addition to the small terminal product, there are bands all throughout the gel that accumulate linearly throughout the 90 min time course.
5. Fig. 5D: The endonuclease activity of the PIN domain is clearly very sensitive to divalent cation type and concentration. Were other conditions tested to more rigorously rule out endonuclease activity for the hDIS3L enzyme?

Referee #2 (Remarks to the Author):

In this manuscript the authors describe two human homologues of the yeast DIS3 protein. In yeast, DIS3 is the catalytic component of the RNA processing and degrading exosome complex. One human homolog has previously been described, but its association with the exosome was not detected. In this manuscript the authors show that humans contain an additional DIS3 homologue, that both homologues associate with the exosome, and that both contribute to exosome activity, both biochemically and in vivo. They also conclude that hDIS3 is mainly nuclear, and hDIS3L exclusively cytoplasmic. These significant conclusions are generally well-supported by the data and merit publication in EMBO. However, there are a number of relatively minor issues that should be addressed.

Major issues:

1. The data that hDIS3L is strictly cytoplasmic is unconvincing. Figure 3D clearly shows a band with the hDIS3L antibody in the cytoplasmic fraction. The figure legend dismisses this as an

unspecific band (which should be nonspecific), but no evidence for this is given. Can the authors rule out that they detect a second isoform here, and that this second isoform is cytoplasmic? In this context it is interesting to note that the accompanying manuscript by Stalls et al reports the possible existence of 4 isoforms of hDIS3L. Most of the other localization data depends on expressing one particular isoform as a tagged protein, and therefore does not address the possibility of a distinct cytoplasmic isoform. If the band detected in the cytoplasmic fraction is indeed nonspecific, it should still be there after hDIS3L knockdown, however that is not what figure 4A shows. Can the authors comment?
On a related note, if the hDIS3L antibody binds to a nonspecific protein, it is not a suitable reagent to localize hDIS3L by immunofluorescence as done in figure 3E.

2. The quantitation of the relative expression levels of hDIS3, hDIS3L and hRRP6 is problematic. The authors do not include detailed methods, but if I understand them right, an underlying assumption they used is that expression of Flag-tagged version of each of these has no effect on the expression level of the endogenous protein, which seems very unlikely.

3. On p7 the authors conclude that the two human DIS3 genes arose by gene duplication "during the course of animal evolution". However, that is not what is suggested by the data shown. if DIS3 and DIS3L arose by gene duplication during animal evolution, one would expect them to be more similar to each other that to fungal or plant DIS3. However the similarity cited in the paper and the tree in figure S1 shows that hDIS3 is more like yeast DIS3 than hDIS3L. Related to the evolutionary history, the Chekanova et al (2000) reported two Arabidopsis DIS3 homologs, however only one is included in the analysis in figure S1. To clarify whether DIS3 was duplicated during animal evolution, or whether DIS3L was lost during fungal evolution, the authors should do a more complete phylogenetic analysis that includes all of the DIS3 homologs for each of the species examined (including hDIS3L2).

Minor comments:

4. On p8 the authors show that the hDIS3L cDNA does not complement a yeast DIS3 mutant. They conclude that "there are significant functional differences between hDIS3 and hDIS3L". This is a grand conclusion based on this one negative result. There are many other explanations why the human cDNA may not complement the yeast mutant. The authors haven't even shown that they successfully expressed the human protein in their yeast strain, even though they have the antibodies to do so.

5. The materials and methods section is incomplete. The methods for knock experiments are missing. The description of various plasmids used is missing. The reference given for the yeast plasmid p415 vector does not describe this plasmid. Does each lane in figure 3D contain nuclear and cytoplasmic fractions from the same number of cells, or was the same amount of total protein loaded?

6. Figure 2B and the supplemental tables appear to conflict since only a subset of identified specific proteins listed in the tables are included in the figure. What reason was used to include or exclude specific proteins?

7. On p14, the authors state that they obtained "relatively pure proteins" (line 8), but the gel clearly shows three major bands. The figure legend states that the other two are "degradation products", but does not explain what data supports that conclusion. Given that there are other proteins present, I think the conclusion that hDIS3-FLAG displays nucleolytic activity should be changed to hDIS3-FLAG copurifies with nucleolytic activity.

8. On p14 5th line from bottom DIS3 and hDIS3L proteins performed exonucleolytic digestion, should be changed to hDIS3 and hDIS3L fractions had nucleolytic activity

9. In several places the authors conclude that hDIS3 and hDIS3L have processive exonucleolytic activity, but they never test whether the activity is exonucleolytic or processive.

10. On p 16 the authors state "we favor the alternative that irrespective of the seemingly weak interaction, hDIS3 and hDIS3L perform their cellular tasks as exosome subunits. This is because a

great portion of exosome core particles would otherwise be left without processive ribonuclease activity." The authors never quantify the expression level of the core exosome, relative to hDIS3 and hDIS3L, and thus can not exclude that there is an excess of the DIS3 homologs over the core exosome. Showing gels of their immunofurifications may address this.

11. On p15 the authors state that "hDIS3DM had no such activity", however some product seems to accumulate by 90 minutes. "strongly reduced" seems a better description of the data than "no"

12. In several places the authors state the human genome encodes two DIS3 homologues, while in fact there are three. (e.g. line 2 of page 7)

13. On p9 line 9 "hybridization" should be changed to "binding"

14. On p7 the phrase "paralogous gene duplication" is redundant since all gene duplications result in paralogs. On p 15 the phrase functional homolog makes little sense. Homology refers to common dissent and does not address whether two proteins have the same function.

15. On p23 "The nuclei were resuspended in S1 buffer (0.25 M sucrose, 10 mM MgCl2) and layered on top of an equal volume of S3 buffer (0.88 M sucrose, 0.5 mM MgCl2)" should be changed to "The nuclei were resuspended in S1 solution (0.25 M sucrose, 10 mM MgCl2) and layered on top of an equal volume of S3 solution (0.88 M sucrose, 0.5 mM MgCl2)". On p 20 and 21 buffer B and buffer A should be changed to solution B and solution A

16. On p4 the authors describe the exosome core as "biochemically idle" The word "idle" implies that the core is capable of doing catalysis, but is not currently doing it. This does not reflect current thinking that the exosome core is incapable of catalysis and therefore idle should be changed to inactive.

Referee #3 (Remarks to the Author):

The authors have identified a novel human exosome component, hDis3L. For the first time they show association of the hDis3 with exosome and also association of hDis3L with this complex. They proved the differential localization of these exosome components and compare it with the localization of RRP6. It should be mentioned that authors used set of different methods which makes their results more accurate. It seems that hDIS3L is entirely cytoplasmic, hDIS3 predominantly nuclear and hRRP6 is not nucleus restricted but it accumulates in the nucleolus. They performed biochemical tests of purified proteins and the in vitro experiments indicated that Dis3 (similarly to its yeast Rrp44(Dis3)counterpart) has both exo and endonucleolytic activities while Dis3L possess only exo activity and an inactive PIN domain.

They give some examples of Dis3 associated RNA substrates and some, but not so clear, evidences of involvement of Dis3L in cytoplasmic RNA degradation. On the other hand they showed that depletion of cytoplasm Dis3L does not seem to influence the metabolism of Dis3 nuclear substrates. It is unfortunate that it has not well proven that hDIS3L has an in vivo function.

I found this paper quite interesting and I think that some conclusions will be very important for all groups studying RNA degradation in eukaryotic cells. The methods used were versatile and appropriate. In general I do not have many remarks, but I think that some controls are needed and some improvements are still required.

Specific Comments:
Attention that both in the running title and the abstract you imply that you have demonstrated that the 3 RNases are the exosome catalytic subunits. "Running title: Catalytic subunits of the exosome and Abstract: last line -three different ribonucleases can serve as catalytic subunits for the exosome in human cells." In your work you only have demonstrated in vitro that the RNases are active. Even though we can deduct this conclusion, it is not completely demonstrated that these the catalytic subunits of the exosome. Accordingly you have to be cautious regarding your conclusions and adapt

them to what you have shown.

It is good that in the abstract you mention that yeast Dis3 is also known as Rrp44 (many papers only mention the designation Rrp44). For the same reason, to be easy to locate your manuscript, I think that the abstract you have to include the word RNase II/ RNase R family of enzymes and/or RNB domain that is characteristic of these family of enzymes.
This should also be taken into account for keywords. The word "endonucleolysis" is not very common in the papers in this area. It is better to mention endonuclease or endonucleolytic cleavage. Please also substitute this throughout the text.

Introduction:
First paragraph- line 6 to line 10 the sentences are confusing- please rearrange.
Page 5 -first line- When you define the RNase II/ RNase R region you have to give the references that have defined them. According ...after 3´-5´exonucleolytic activity. add the 2 references 1- Frazão C, McVey CE, Amblar M, Barbas A, Vonrhein C, Arraiano CM, Carrondo MA. Unravelling the dynamics of RNA degradation by ribonuclease II and its RNA-bound complex. Nature. 2006 Sep 7;443(7107):110-4.
And 2- Zuo Y, Vincent HA, Zhang J, Wang Y, Deutscher MP, Malhotra A.
Structural basis for processivity and single-strand specificity of RNase II.
Mol Cell. 2006 Oct 6;24(1):149-56. Epub 2006 Sep 21.

Page 5, line 10 after ...individual abolition of the nucleolytic activities only leads to decreased growth.-include the references (Lebreton et al, 2008; Schaeffer et al, 2009; Schneider et al, 2009).

The human genome encodes two homologs of yeast Rrp44/Dis3

In fact, there are 3 genes homologous to yeast Dis3p. They should say that they only want to focus in 2 genes. Was the third present in any co-immunoprecipitation with the human core exosome (using SILAC followed by MS)?

FIG 1- Show the linear domain organization of the yeast vRrp44/Dis3 versus the two human Dis3 proteins. Enlarge a little the aminoacids.
FIG.1-D How long have you left the strains grow in -LEU +DOX?

I also think they should give the GenBank accession number. This way we would be able to compare with previous results.

It would be quite good to show that there is active transcription of the hDIS3L protein.

hDIS3and hDIS3L both co-immunoprecipitate with the human core exosome

This is an interesting result, since they give an answer to an old question. They have finally shown that hDIS3 weakly associates with the exosome.

Why do you think hDis3 only binds to the exosome in less stringent conditions than hDis3L?

Fig2B Can you give an explanation why does hRRP6 comes with the hDIS3-CoIP and the hDIS3L-CoIP?

Figure 2: Can you give any ideas to try to explain the reason why RRP41 co-precipitates only with hDIS3L while hDIS3 co-precipitates with RRP41? The two should appear in the fraction corresponding to the co-immunoprecipitation with RRP41, right ? Please refer to this in the text.

Differential subcellular localization of hDIS3 and hDIS3L

page10- line 7-Be cautions:...we conclude that under these conditions HDIS3 is nuclear...

Page 11: It is reported that hDIS3L is located only in the citoplasm. But in Fig 3D it is possible to

observe a band in the nucleus, a band of lower molecular weight. What is this band? What do you think it is the meaning of this unspecific band detected in the nucleus by the hDIS3L antibody?

How can you affirm that hDIS3 is mainly nuclear if in Fig 3D it looks the opposite?

The in vivo impact of the three human exosome-associated nucleases

Based on the experiments using RNAi silencing , I would like to ask the authors to report what is the impact of the different RNases on cell viability. Since deletion of the S. cerevisiae Rrp44/Dis3 is lethal, I wonder what is happening with the cells when the authors lower the levels of all 3-5` exos that are suggested to cooperate with the exosome in human cells (Dis3 Dis3L and RRP6). The effect should be similar to the one obtained by lowering the level of exosome components, which is known to cause measurable growth rate defects (van Dijk; Pruijn 2007; RNA).

Fig 4A- Why do you think that 40 almost disappears with the knockdown of hRRP6 or hDIS3L and not hDIS3?

In figure 4D they should show the other combination double mutants (hRRP6/hDIS3L and hDIS3/hDIS3L).
Since 6/3 presented difference in the PROMPT in the nucleus and 6/3L did not, it is quite important to show the action of 3L in the cytoplasm. 6/ 3 there is little in the cytoplasm, therefore it is quite important to show the effect of 6/3L.
In the cytoplasm it is also important to see the effect of the two exos: 3/3l in the cytoplasm.

Can you propose an explanation why the knock down of hRRP40 depletes hRRP6 and hDIS3L but not hDIS3?

Both hDIS3 and hDIS3L are exonucleases but only hDIS3 displays PIN-domain dependent endonucleolytic activity

In the protein purification how do you know that the lower bands are degradation products and not another protein that has co-purified? Moreover, in most cases the "degradation product"! exists in a larger amount than the purified protein.

What is the reason why in the immunoprecipitation you elute hRRP41 with the FLAG peptide but for hDIS3 and hDis3L you use SDS. Why if all of them have the FLAG tag?

hDIS3L purified from E. coli is not active but if it is purified from the human cells it is active --> Do you have any indications of any post-translational modification?

Page 15 , line 3 after activity... of the PIN domain add the references (Lebreton et al, 2008; Schaeffer et al, 2009; Schneider et al, 2009).

In the publication of Lebreton et al 2008, it was given the reasoning why the substrates and buffer conditions were appropriate to show the activity of PIN. However it would be easier to read this paper if it was made clear in the text why the substrates chosen could only be cleaved with an active PIN, with an active endonuclease.

Figure 5: The activity gel of hDIS3L presents many intermediate bands that are not present in the gel of hDIS3. If the substrate is the same why does this happen only with one of the proteins?
In none of the gels it is referred the size of the final fragment that is liberated by the enzyme. Is it similar to what happens with yeast RRp44?
You never refer the amount of protein used in the assays, not in the respective figure not in the legends.
Figure 5A - can hardly see hDIS3L. Besides, figure is too small.
Figure 5B, C e D - didn't put protein concentration.
Fig5B It has to be referred in the text that when we compare hDIS3L shows much less

exonucleolytic activity than hDIS3.

If hDIS3L does not have any endonucleolytic activity why do you think that this protein still has the PIN domain? Could it be involved with the attachment to the exosome?
Do you have any mutants to test this and add the conclusion in the text?

Discussion:

Page 16 Line 5 It is said that hDIS3 and hDIS3L physically interact with the exosome, but are they really the catalytic subunits? If we take the activity of hDIS3 and/or hDIS3L is the exosome active? This is not so clear when it is compared with the knock down of hRRP40...

Page 16 line 5 from the end: ..."depleting both nuclear catalytic subunits hDIS3 and hRRP6." Correc this taking into account that it is known that both hDIS3 and hRRP6 can also be cytoplasmic even though they are more abundant in the nucleus.

Page 17: line 14_ please explain better what you want to say...

Page 17 2nd paragraph. The sentence is exaggerated: The only contrast with yeast is that hRRP6 also seems to be cytoplasmic...

1st Revision - authors' response 11 May 2010

Referee #1:

1. Supplementary Fig. 4: Given that the anti-DIS3L antibody detects a non-specific, ~co-migrating band in western blots (Fig. 3D) as well as innate difficulties in normalizing western blot efficiencies of individual antibodies, it is not clear to me how truly quantitative and thus meaningful is the analysis presented in Fig. S4.

The non-specific band detected by the anti-DIS3L antibody in the original Fig. 3D is not reproducible. It was only observed once due to the usage of a low quality antibody batch. The experiment has been repeated several times with new and better antibody batches and in all these experiments only one hDIS3L band is detected. The new Fig. 4D contains a representative result from these new fractionation analyses. In any case, the band was only seen originally when probing nuclear extract by western analysis. Thus, the issue has no bearing anyway on the westerns of the total cell extract in Fig. S4.
In Fig. S4 we do not simply cross-compare signals produced by individual antibodies. Rather, we use antibodies directed against the individual endogenous proteins to determine their degree of overexpression (levels of endogenous protein + the FLAG-fusion). Hereafter, relative levels of the factors are compared by anti-FLAG westerns, where the obvious assumption is that the respective FLAG fusions are recognized with equal efficiencies by the anti-FLAG antibody. Although we fully agree that this type of experiment is semi-quantitative and can only serve as a rough guideline, we believe it offers an initial characterization of the relative stoichiometries of these factors in human

cells. However, to accommodate the reviewer, we have now treated the results accordingly cautiously in the text.

2. Fig. 5 and elsewhere - please clearly define the ss17-A14 and related substrates for the reader

Done.

3. Figs. 5. S5 and S6: If I understand the experiment correctly, these studies were performed with 5' labeled RNA substrates. Therefore this assay alone does not rule out endo activities (or 5'-3' exonuclease activity for that matter). While I think these alternative activities are unlikely given what has been previously published in yeast, nonetheless this is the first time the activities of these two human proteins will be presented. Thus I feel it is important for the authors to clearly demonstrate for the reader that they are revealing a 3'-5' exonuclease. For example, does a 3' structure block the nuclease of the protein? Does the protein have activity on circular substrates?

All biochemical experiments of the paper were redone. This time using yeast Dis3p, which was previously shown to harbour processive 3'-5' exoribonuclease activity, as a positive control. Degradation assays were performed employing both 5'- and 3'-labeled substrates (Figure 6B; Supplementary Figure S8). In all these experiments the three enzymes (yeast Dis3p, hDIS3 and hDIS3L) behaved similarly: the major degradation product for the 3'-labeled substrate is a mononucleotide, while final products of the 5'-labeled substrate are 4-5 nucleotides long. These patterns can only be easily explained by exonucleolytic degradation initiating from the 3'-end. Endonucleolytic activity would yield accumulation of a ladder of products, similarly to what we observe for the hDIS3 PIN domain (Figure 7A; Supplementary Figure S9), and 5'-3' exoribonucleolytic activity would yield a mononucleotide as the major degradation product with the 5'-labeled substrate. Taken together, our data strongly support 3'-5' exonucleolytic activity. These arguments have now been incorporated into the text.

4. Fig. 5B: The data using the hDIS3L-TAP is not very convincing. In addition to the small terminal product, there are bands all throughout the gel that accumulate linearly throughout the 90 min time course.

Our new hDIS3- and hDIS3L-TAP purifications were much cleaner (new Figure 6A) and consequently the background nucleolytic degradation was lowered significantly (new Figure 6B). Moreover, a new negative control was included where we performed enzymatic assays with mock fractions purified from HEK293 Flp-In T-Rex cells transfected with an empty pcDNA5/FRT/TO vector.

5. Fig. 5D: The endonuclease activity of the PIN domain is clearly very sensitive to divalent cation type and concentration. Were other conditions tested to more rigorously rule out endonuclease activity for the hDIS3L enzyme?

We have now tested several different concentrations of various divalent cations in a new Supplementary Figure S10. We were unable to detect endoribonucleolytic activity of the hDIS3L protein, irrespective of the reaction conditions.

Referee #2:

1. The data that hDIS3L is strictly cytoplasmic is unconvincing. Figure 3D clearly shows a band with the hDIS3L antibody in the cytoplasmic fraction. The figure legend dismisses this as an unspecific band (which should be nonspecific), but no evidence for this is given. Can the authors rule out that they detect a second isoform here, and that this second isoform is cytoplasmic?

We take it that the reviewer refers to the non-specific band of the "nuclear" and not the "cytoplasmic" fraction of the original Fig. 3D. As explained above (Reviewer #1, point #1), appearance of this band is not reproducible and was likely the result of background detection by a low quality antibody batch used in this particular experiment. In several additional experiments, using better hDIS3L antibody batches, we have not seen evidence of potential hDIS3L isoforms at the protein level, neither in nuclear nor cytoplasmic extracts. While this does not prove that hDIS3L isoforms do not exist, they are certainly not detected by the available polyclonal antibody, which was produced using most of the full length protein (residues 84-1054 of 1054) as an antigen (see also below).

In this context it is interesting to note that the accompanying manuscript by Stalls et al reports the possible existence of 4 isoforms of hDIS3L. Most of the other localization data depends on expressing one particular isoform as a tagged protein, and therefore does not address the possibility of a distinct cytoplasmic isoform.
If the band detected in the cytoplasmic fraction is indeed nonspecific, it should still be there after hDIS3L knockdown, however that is not what figure 4A shows. Can the authors comment?

As explained above, we have only seen one distinct hDIS3L species by western blotting, a band which consistently disappear upon specific hDIS3L siRNA administration. To our knowledge, Staals and colleagues do not specify the nature of the other three potential isoforms of DIS3L. We assume the four cDNAs in question are the ones containing poly(A) tails, that can be found in the GeneBank database. One of these encodes the protein we believe is the functional form of DIS3L with a predicted molecular weight compatible with the size we detect using the hDIS3L antibody. The other "isoforms" would encode N-terminally truncated proteins. The shortest one, BC014124, would lack the PIN and CSD domains, which would render the protein non-functional. Notably, this cDNA is annotated as a "partial cDNA" and therefore the encoded protein should not be considered a valid isoform of DIS3L. The other two, BC022089 and NM_133375, contain open reading frames

starting at the M84 position of the full length protein which would disrupt the PIN domain. Since in yeast Dis3p this domain is indispensable for interaction with the exosome core, a truncated variant of DIS3L would not be able to form the interaction with the exosome that we observe. In addition, it is very rare that proteins begin or terminate in the middle of folded domains of their functional isoforms. Thus, in our opinion all the other cDNAs are either artefactual or do not yield stable protein products *in vivo*.

On a related note, if the hDIS3L antibody binds to a nonspecific protein, it is not a suitable reagent to localize hDIS3L by immunofluorescence as done in figure 3E.

The specificity of the hDIS3L IF signal using this reagent was controlled by its disappearance upon hDIS3L depletion by RNAi. This point is now mentioned in the text.

2. The quantitation of the relative expression levels of hDIS3, hDIS3L and hRRP6 is problematic. The authors do not include detailed methods, but if I understand them right, an underlying assumption they used is that expression of Flag-tagged version of each of these has no effect on the expression level of the endogenous protein, which seems very unlikely.

As explained above (Reviewer #1, point #1), these data are based on over-expression measurements with separate “endogenous” antibodies followed by an assessment of relative levels using the FLAG-antibody against this common epitope of the protein fusions. This experimental outline is now stated clearer in the Fig. S4 legend. We agree that these data merely serve as a rough guideline for relative stoichiometries and have therefore toned down the corresponding conclusions in the text.

3. On p7 the authors conclude that the two human DIS3 genes arose by gene duplication "during the course of animal evolution". However, that is not what is suggested by the data shown. if DIS3 and DIS3L arose by gene duplication during animal evolution, one would expect them to be more similar to each other that to fungal or plant DIS3. However the similarity cited in the paper and the tree in figure S1 shows that hDIS3 is more like yeast DIS3 than hDIS3L. Related to the evolutionary history, the Chekanova et al (2000) reported two Arabidopsis DIS3 homologs, however only one is included in the analysis in figure S1. To clarify whether DIS3 was duplicated during animal evolution, or whether DIS3L was lost during fungal evolution, the authors should do a more complete phylogenetic analysis that includes all of the DIS3 homologs for each of the species examined (including hDIS3L2).

With regard to the phrase “during the course of animal evolution”, we agree with the reviewer. The presented data do not support a recent gene duplication event. Therefore, we have removed the word “animal”.
The *Arabidopsis* genome indeed encodes two proteins homologous to Dis3. However, only one of these is predicted to contain all Dis3 domains including the PIN domain. In this paper, we focus on

proteins that can interact with the exosome. Therefore, the other *Arabidopsis* homologue of Dis3 is excluded from the phylogenetic analysis. For the same reason the vertebrate Dis3-like 2 (Dis3L2) proteins were not included in the phylogenetic and experimental analysis. These issues are now explained in the text.

4. On p8 the authors show that the hDIS3L cDNA does not complement a yeast DIS3 mutant. They conclude that "there are significant functional differences between hDIS3 and hDIS3L". This is a grand conclusion based on this one negative result. There are many other explanations why the human cDNA may not complement the yeast mutant. The authors haven't even shown that they successfully expressed the human protein in their yeast strain, even though they have the antibodies to do so.

In order to exclude the possibility that the lack of complementation of yeast *dis3* by hDIS3L is caused by expression problems, we examined both RNA and protein levels (new Figure 2). This demonstrated the presence of both hDIS3 and hDIS3L transcripts in total RNA preps from the yeast subjected to complementation assays (Figure 2B). Since we were unable to detect hDIS3 and hDIS3L proteins using “endogenous” antibodies, we turned to vectors expressing FLAG-tagged versions, which yielded similar results in the complementation assays (bottom part of Figure 2A). In this case, in addition to showing active transcription by northern blotting analysis (Figure 2B), we were able to also show the presence of protein by anti-FLAG westerns (Figure 2C) and by mass spectrometry. All these results are now included in the manuscript.

5. The materials and methods section is incomplete. The methods for knock experiments are missing. The description of various plasmids used is missing. The reference given for the yeast plasmid p415 vector does not describe this plasmid. Does each lane in figure 3D contain nuclear and cytoplasmic fractions from the same number of cells, or was the same amount of total protein loaded?

We are puzzled about the remark on the missing plasmid descriptions. Pehaps the reviewer could specify?
A description of the depletion experiments has now been added. The reference describing p415 vector has been corrected. Similar cell equivalents of nuclear and cytoplasmic extracts were loaded in the new Figure 4D. This is now clarified in the figure legend.

6. Figure 2B and the supplemental tables appear to conflict since only a subset of identified specific proteins listed in the tables are included in the figure. What reason was used to include or exclude specific proteins?

We thank the reviewer for spotting these inconsistencies. In the new Figure 3B only proteins with a SILAC ratio above background (as defined in the Materials and Methods) are shown. Moreover, proteins detected by only one peptide are omitted unless they are known exosome components, in

which case they are marked by an asterisk. However, in the interest of space we have chosen to cut the list of interactors after the last identified exosome component, leaving the rest of the information in the supplemental tables. This is now clarified in the figure legend.

7. On p14, the authors state that they obtained "relatively pure proteins" (line 8), but the gel clearly shows three major bands. The figure legend states that the other two are "degradation products", but does not explain what data supports that conclusion. Given that there are other proteins present, I think the conclusion that hDIS3-FLAG displays nucleolytic activity should be changed to hDIS3-FLAG copurifies with nucleolytic activity.

As described above (Reviewer #1, points #3-4), all proteins used for biochemical analysis were re-purifed with much cleaner results. We also analyzed potential contaminants/degradation products by mass spectrometry. They turned out to be light and heavy chains of antibodies from the anti-FLAG resin. This is now clarified in the new Supplementary Figure S7 legend. Our previous conclusion was based on western blotting analysis which misled us in our initial interpretation of the data.

8. On p14 5th line from bottom DIS3 and hDIS3L proteins performed exonucleolytic digestion, should be changed to hDIS3 and hDIS3L fractions had nucleolytic activity

Done.

9. In several places the authors conclude that hDIS3 and hDIS3L have processive exonucleolytic activity, but they never test whether the activity is exonucleolytic or processive.

The newly added data shows that hDIS3, hDIS3L and yeast Dis3 produce similar degradation patterns of 5'- as well as 3'-labeled substrates (see Reviewer #1, point #3), which strongly argues for 3'-5' exonucleolytic activity of these enzymes. Regarding the issue of processive versus distributive activity, we mostly observe final products in our biochemical assays, while the accumulation of intermediates is very low. This strongly suggests that the 3'-5' exoribonucleolytic activity of the RNB domain is processive as is the case for yeast Dis3p, which was used in parallel as a control.

10. On p 16 the authors state "we favor the alternative that irrespective of the seemingly weak interaction, hDIS3 and hDIS3L perform their cellular tasks as exosome subunits. This is because a great portion of exosome core particles would otherwise be left without processive ribonuclease activity." The authors never quantify the expression level of the core exosome, relative to hDIS3 and hDIS3L, and thus can not exclude that there is an excess of the DIS3 homologs over the core exosome. Showing gels of their immunofurifications may address this.

Since interactions between the exosome core and hDIS3/hDIS3L are of low affinity and possibly of transient nature, we do not think that SDS-PAGE analysis of the immunopurifications would answer this question. By the above mentioned statement, we did not mean to imply that cells cannot harbor “catalytically empty core exosomes”, let alone conclude anything concerning possible combinations of complexes. This will be a non-trivial question to answer and is beyond the scope of the present paper. We simply wanted to state that in the degradation of the nuclear PROMPT substrates it takes depletion of both hRRP6 and hDIS3 to reach a stabilization level that compares to the one obtained by depleting a core component (hRRP40). Our interpretation is that, at least for these substrates, hDIS3 seems to work in the context of the exosome. The sentence has now been modified to emphasize this point.

11. On p15 the authors state that "hDIS3DM had no such activity", however some product seems to accumulate by 90 minutes. "strongly reduced" seems a better description of the data than "no"

Experiments were repeated and now the background nucleolytic activity is significantly lower and similar to the new negative control. Therefore we would prefer to keep the sentence as it is.

12. In several places the authors state the human genome encodes two DIS3 homologues, while in fact there are three. (e.g. line 2 of page 7)

This has been addressed above (Reviewer #2, point #3). A short note about the hDIS3L2 protein and the reason for its omission has been added to the manuscript.

13. On p9 line 9 "hybridization" should be changed to "binding"

Done.

14. On p7 the phrase "paralogous gene duplication" is redundant since all gene duplications result in paralogs. On p 15 the phrase functional homolog makes little sense. Homology refers to common dissent and does not address whether two proteins have the same function.

The reviewer is correct. The phrase “paralogous gene duplication” has been corrected and the phrase “functional homolog” has been removed.

15. On p23 "The nuclei were resuspended in S1 buffer (0.25 M sucrose, 10 mM MgCl2) and layered on top of an equal volume of S3 buffer (0.88 M sucrose, 0.5 mM MgCl2)" should be changed to "The nuclei were resuspended in S1 solution (0.25 M sucrose, 10 mM MgCl2) and layered on top of an equal volume of S3 solution (0.88 M sucrose, 0.5 mM MgCl2)". On p 20 and 21 buffer B and buffer A should be changed to solution B and solution A

Done.

16. On p4 the authors describe the exosome core as "biochemically idle" The word "idle" implies that the core is capable of doing catalysis, but is not currently doing it. This does not reflect current thinking that the exosome core is incapable of catalysis and therefore idle should be changed to inactive.

Done.

Referee #3:

Attention that both in the running title and the abstract you imply that you have demonstrated that the 3 RNases are the exosome catalytic subunits. "Running title: Catalytic subunits of the exosome and Abstract: last line -three different ribonucleases can serve as catalytic subunits for the exosome in human cells." In your work you only have demonstrated in vitro that the RNases are active. Even though we can deduct this conclusion, it is not completely demonstrated that these the catalytic subunits of the exosome. Accordingly you have to be cautious regarding your conclusions and adapt them to what you have shown.

We have softened the title to read “The human core exosome interacts with differentially localized processive RNases: hDIS3 and hDIS3L”, the running title to be: “Ribonucleases interacting with the human exosome” and the last line of the abstract to: “Our data suggest that three different….”.

… to be easy to locate your manuscript, I think that the abstract you have to include the word RNase II/ RNase R family of enzymes and/or RNB domain that is characteristic of these family of enzymes. This should also be taken into account for keywords.

The abstract now reads: “The former is a nuclear and cytoplasmic RNaseII/R-like enzyme…….while the latter is a distributive RNase D-like nuclear exonuclease”.
As extra keywords we have added “RNB domain” and “RNaseII/R enzymes”

The word "endonucleolysis" is not very common in the papers in this area. It is better to mention endonuclease or endonucleolytic cleavage. Please also substitute this throughout the text.

Done.

Introduction:
First paragraph- line 6 to line 10 the sentences are confusing- please rearrange.

This has been rephrased.

Page 5 -first line- When you define the RNase II/ RNase R region you have to give the references that have defined them. According ...after 3´-5´exonucleolytic activity. add the 2 references 1- Frazão C, McVey CE, Amblar M, Barbas A, Vonrhein C, Arraiano CM, Carrondo MA. Unravelling the dynamics of RNA degradation by ribonuclease II and its RNA-bound complex. Nature. 2006 Sep 7;443(7107):110-4.
And 2- Zuo Y, Vincent HA, Zhang J, Wang Y, Deutscher MP, Malhotra A.
Structural basis for processivity and single-strand specificity of RNase II.
Mol Cell. 2006 Oct 6;24(1):149-56. Epub 2006 Sep 21.

Done.

Page 5 , line 10 after ...individual abolition of the nucleolytic activities only leads to decreased growth.-include the references (Lebreton et al, 2008; Schaeffer et al, 2009; Schneider et al, 2009).

Done.

In fact, there are 3 genes homologous to yeast Dis3p. They should say that they only want to focus in 2 genes. Was the third present in any co-immunoprecipitation with the human core exosome (using SILAC followed by MS)?

We have now stated in the text, which genes we focus on. No, we did not detect hDIS3L2 in any of the co-immunoprecipitations.

FIG 1- Show the linear domain organization of the yeast vRrp44/Dis3 versus the two human Dis3 proteins. Enlarge a little the aminoacids.

Figure 1 has been modified according to the reviewer's suggestion. We also included domain organization of *E. coli* RNase II for reference.

FIG.1-D How long have you left the strains grow in -LEU +DOX?

The strains were left in -LEU +DOX media for 60 h. This information has been included both in the methods section and in the new Figure 2 legend.

I also think they should give the GenBank accession number. This way we would be able to compare with previous results.

Done.

It would be quite good to show that there is active transcription of the hDIS3L protein.

As described above (Reviewer #2, point #4), the expression of hDIS3 and hDIS3L has now been verified by northern and western blot analyses as well as by mass spectrometry.

Why do you think hDis3 only binds to the exosome in less stringent conditions than hDis3L?

Our initial pull down experiments using hDis3 protein as a bait indicated that in order to see core exosome components coming down we needed to lower the salt concentration, which was not the case for hDIS3L. We can only speculate what properties of these proteins are responsible for such a difference in affinities and at this stage it would be premature to add such speculations to the manuscript.

Fig2B Can you give an explanation why does hRRP6 comes with the hDIS3-CoIP and the hDIS3L-CoIP?

The most straightforward explanation is that the exosome core (or some exosomes core particles) is capable of interacting with hRRP6 and hDIS3 or hDIS3L simultaneously. This idea is also illustrated in the model in Figure 8.

Figure 2: Can you give any ideas to try to explain the reason why RRP41 co-precipitates only with hDIS3L while hDIS3 co-precipitates with RRP41? The two should appear in the fraction corresponding to the co-immunoprecipitation with RRP41, right ? Please refer to this in the text.

In order to detect hDIS3/exosome subunit interactions we had to lower the salt concentration of the hDIS3 co-IP from 100 mM NaCl to 75 mM NaCl. In contrast the hDIS3L/exosome interaction is detected at 100 mM NaCl independent on whether hDIS3L or hRRP41 is the bait. These issues are explained in the text.

page10- line 7-Be cautions:...we conclude that under these conditions HDIS3 is nuclear...

This has been rephrased.

Page 11: It is reported that hDIS3L is located only in the citoplasm. But in Fig 3D it is possible to observe a band in the nucleus, a band of lower molecular weight. What is this band? What do you think it is the meaning of this unspecific band detected in the nucleus by the hDIS3L antibody?

As explained above (Reviewer #1, point #1), appearance of this band is not reproducible.

How can you affirm that hDIS3 is mainly nuclear if in Fig 3D it looks the opposite?

We do not agree that Fig. 3D looks "the opposite". In fact our new fractionation data (new Figure 4D) detect more hDIS3 in the nucleus than in the cytoplasm. However, the method is not perfect and for hDIS3 localization it has been subject to slight variation, which is now stated in the text. In contrast, the fractionation experiments consistently yield exclusively cytoplasmic hDIS3L. This is why we have taken care in calling only hDIS3L a strict "cytoplasmic factor", while hRRP6 and hDIS3 are stated to reside in both compartments, but to be "mostly" nuclear.

Based on the experiments using RNAi silencing , I would like to ask the authors to report what is the impact of the different RNases on cell viability. Since deletion of the S. cerevisiae Rrp44/Dis3 is lethal, I wonder what is happening with the cells when the authors lower the levels of all 3-5` exos that are suggested to cooperate with the exosome in human cells (Dis3 Dis3L and RRP6). The effect should be similar to the one obtained by lowering the level of exosome components, which is known to cause measurable growth rate defects (van Dijk; Pruijn 2007; RNA).

As a new Supplementary Figure S5 we have included cell counts 2 and 4 days after the second siRNA administration of the various depletion experiments. In a somewhat surprising contrast to the situation reported for HepG2 cells (van Dijk; Pruijn 2007), there is basically no impact on growth after these respective depletions. We do not know whether this relates to incomplete knock downs leaving enough residual exosome activity to support growth or whether Hela cells are able to compensate for, or are less dependent on, exosome activity than HepG2 cells. These issues are now mentioned in the text.

Fig 4A- Why do you think that 40 almost disappears with the knockdown of hRRP6 or hDIS3L and not hDIS3?

This must be a misunderstanding. This figure shows that hRRP40 levels are rather constant unless siRNAs against its own mRNA is administered.

In figure 4D they should show the other combination double mutants (hRRP6/hDIS3L and hDIS3/hDIS3L).
Since 6/3 presented difference in the PROMPT in the nucleus and 6/3L did not, it is quite important to show the action of 3L in the cytoplasm. 6/ 3 there is little in the cytoplasm, therefore it is quite important to show the effect of 6/3L.
In the cytoplasm it is also important to see the effect of the two exos: 3/3l in the cytoplasm.

We have now repeated the *c-MYC* RNA analysis as requested. We have also included in the new Figure 5D an analysis of the *c-FOS* RNA, which exhibits a similar trend.

Can you propose an explanation why the knock down of hRRP40 depletes hRRP6 and hDIS3L but not hDIS3?

Although not thoroughly examined, the co-IPs suggest a lower affinity interaction of hDIS3 with the exosome core than for hRRP6 and hDIS3L. Perhaps therefore the two latter factors are more significantly affected by the core subunit depletion than the former.

In the protein purification how do you know that the lower bands are degradation products and not another protein that has co-purified? Moreover, in most cases the "degradation product"! exists in a larger amount than the purified protein.

This issue has been addressed above (Reviewer #2, point #7).

What is the reason why in the immunoprecipitation you elute hRRP41 with the FLAG peptide but for hDIS3 and hDis3L you use SDS. Why if all of them have the FLAG tag?

The most optimal ways of eluting were determined empirically. We do not know why hRRP41 is more readily eluted with FLAG peptide than hDIS3 and hDIS3L. However, this observation is by no means unusual.

hDIS3L purified from E. coli is not active but if it is purified from the human cells it is active --> Do you have any indications of any post-translational modification?

No. However, we should point out that we were not able to purify full-length recombinant hDIS3L from *E. coli* and obtained only the C-terminal part of this protein without its PIN domain. In yeast, the lack of the PIN domain seems to be dispensable for efficient exoribonuclease activity of Dis3, at least for single-stranded substrates (Lorentzen et al., 2008). However, we do not know at this stage whether the same is true for hDIS3L.

Page 15 , line 3 after activity... of the PIN domain add the references (Lebreton et al, 2008; Schaeffer et al, 2009; Schneider et al, 2009).

Done.

In the publication of Lebreton et al 2008, it was given the reasoning why the substrates and buffer conditions were appropriate to show the activity of PIN. However it would be easier to read this

paper if it was made clear in the text why the substrates chosen could only be cleaved with an active PIN, with an active endonuclease.

New experiments have been added, where we employ substrates labeled in different ways to demonstrate the endonucleolytic activity of hDIS3 PIN domain more convincingly. The text has been modified to emphasize the rationale behind choosing these substrates.

Figure 5: The activity gel of hDIS3L presents many intermediate bands that are not present in the gel of hDIS3. If the substrate is the same why does this happen only with one of the proteins?

The repeated purification was more efficient and the mentioned intermediates have essentially disappeared (see also Reviewer #1, point #4).

In none of the gels it is referred the size of the final fragment that is liberated by the enzyme. Is it similar to what happens with yeast RRp44?

We have now included assays using yeast Dis3 as a control and a 3 nucleotides molecular weight marker. This has allowed us to demonstrate that hDIS3/hDIS3L 3'-5' exoribonuclease activities produce 4-5 nt long final degradation products, similarly to the yeast enzyme. This is now pointed out in the text.

You never refer the amount of protein used in the assays, not in the respective figure not in the legends.

Protein amounts have now been estimated by densitometry of Coomassie-stained SDS-PAGE gels using serial dilutions of BSA as a standard. Concentrations are included in the figure legends.

Figure 5A - can hardly see hDIS3L. Besides, figure is too small.

hDIS3L is now clearly visible in the new Figure 6A. Pictures of the SDS-PAGE gels have been enlarged.

Figure 5B, C e D - didn't put protein concentration.

The protein concentration is added to the legend.

Fig5B It has to be referred in the text that when we compare hDIS3L shows much less exonucleolytic activity than hDIS3.

With the new protein purifications there are no dramatic differences between the exoribonucleolytic activities of hDIS3L and hDIS3.

If hDIS3L does not have any endonucleolytic activity why do you think that this protein still has the PIN domain? Could it be involved with the attachment to the exosome?
Do you have any mutants to test this and add the conclusion in the text?

As discussed in the manuscript, the PIN domain is essential for interaction of Dis3 proteins with the exosome core, which explains its function in hDIS3L, even though it is catalytically inactive. However, we have not tested any deletion mutants.

Page 16 Line 5 It is said that hDIS3 and hDIS3L physically interact with the exosome, but are they really the catalytic subunits? If we take the activity of hDIS3 and/or hDIS3L is the exosome active? This is not so clear when it is compared with the knock down of hRRP40...

The activity of hRRP6 and the likely functional redundancy between the three assumed catalytic subunits of the human exosome must be taken into account. Therefore, in our opinion, double knock down of hDIS3/hRRP6 or triple knock down of hDIS3/hDIS3L/hRRP6 should give similar phenotypes for the nuclear and cytoplasmic substrates, respectively, as the knock down of hRRP40. This is indeed mostly the case.

Page 16 line 5 from the end: ..."depleting both nuclear catalytic subunits hDIS3 and hRRP6."
Correc this taking into account that it is known that both hDIS3 and hRRP6 can also be cytoplasmic even though they are more abundant in the nucleus.

We have deleted “nuclear catalytic subunits”.

Page 17: line 14_ please explain better what you want to say...

The sentence has been rephrased.

Page 17 2nd paragraph. The sentence is exaggerated: The only contrast with yeast is that hRRP6 also seems to be cytoplasmic...

The sentence has been rephrased.

2nd Editorial Decision 17 May 2010

I have looked through your revised manuscript, your responses to the original reports and I have also discussed them with one of the referees, we find that you have satisfactorily addressed all the concerns raised (see below). I am happy to accept the manuscript for publication in The EMBO Journal. I believe that this will

make a great contribution. You will receive the original acceptance letter in the next day or so.

Yours sincerely

Editor
The EMBO Journal

Referee #1

The authors have carefully responded to the points raised in the original three reviews. I find the revised manuscript to be much improved.

